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A b s t r a c t

Usutu virus (USUV) and West Nile virus (WNV) are emerging pathogens that can cause neurological disease in humans. From March 2012 to June 2013, a sero-survey on wild birds was carried out to investigate the circulation of both viruses in Northwest (NW) Italy. Samples belonging to 47 different bird species have been collected using a volunteer based network and a wildlife rehabilitation center. Four of 297 serum samples had neutralizing antibodies against USUV (P = 1.34%, IC 95% 0.36–3.4), while 10 of 233 samples tested positive for WNV (P = 4.29%, IC 95% 2.07–7.75). Neutralizing antibodies for WNV were significantly more prevalent ($p < 0.001$) in trans-Saharan migrants (P = 21%, IC 95% 9.55–37.3) than in resident and short-distance birds, but no migratory habit-related differences were found for USUV. Antibodies in resident bird species suggest that both viruses are circulating in NW Italy.

1. Introduction

Usutu virus (USUV) and West Nile virus (WNV) are emerging neuro-pathogenic agents that belong to the Japanese encephalitis virus antigenic complex of the family *Flaviviridae*, genus *Flavivirus*. Both viruses are maintained in the environment through a bird-mosquito life cycle (Hubálek, 2008) whereas mammals including humans are so far regarded as incidental or dead-end hosts. Migratory birds are assumed to have a key role in the amplification and circulation of these viruses (Malkinson and Banet, 2002; Weissenböck et al., 2002). WNV appeared for the first time in Italy in 1998 causing encephalitis in horses in Tuscany (Autorino et al., 2002). Ten years later, WNV reappeared in northern Italy affecting horses and humans (Calistri et al., 2010a,b; Monaco et al., 2010, 2011). In order to monitor and control WNV circulation, a serological, entomological and virological surveillance program for West Nile neuroinvasive disease has been implemented at the national level by the Italian Ministry of Health (Italian Ministry of Health, 2008). Within this framework it was possible to detect viral circulation in birds, mosquitoes and equids (Monaco et al., 2010, 2011; Calzolari et al., 2010; Savini et al., 2012, 2013) and several human cases were reported (Calistri et al., 2010a; Rizzo et al., 2009, 2012; Bagnarelli et al., 2011; Delbue et al., 2014). USUV was first reported in Austria in 2001 when a considerable die-off of Eurasian blackbirds (*Turdus merula*) was observed in and around Vienna, but a recent retrospective analysis of archived samples from dead birds in the Tuscany region (Italy) in 1996 provided evidence for an earlier introduction into Europe (Weissenböck et al., 2002, 2013). USUV has been noticed again in the last decade in some regions of northern Italy by virological and serological methods (Calzolari et al., 2013; Lelli et al., 2008;

Savini et al., 2011). In 2009, two cases of human encephalitis associated to USUV infection were reported in Emilia Romagna region (northern Italy) confirming the zoonotic potential of this virus (Cavrini et al., 2009; Pecorari et al., 2009).

By working in collaboration with organizations caring for wildlife the surveillance program may be improved by increasing the number and diversity of samples (Nemeth et al., 2007). Moreover, wildlife rehabilitation centers may greatly enhance and simplify surveillance efforts for avian-related viruses in some areas by concentrating many samples in limited space (Nemeth et al., 2007). Samples collected from free-ranging birds during ringing campaigns may also provide additional epidemiological information (Komar, 2000). In this perspective, we performed a serological investigation within wild birds collected in the Piedmont region in order to investigate the circulation of WNV and USUV viruses. Furthermore, we evaluated how the use of serological investigation from wild birds, obtained by volunteer networks, may integrate the data derived from official surveillance protocols.

2. Materials and methods

2.1. Study area and sampling sites

The study was carried out in Piedmont, a region of Northwest (NW) Italy (Fig. 1A). From March 2012 to June 2013, 304 blood samples were collected from wild birds belonging to 47 different species (Table 1A and B). Of these, 168 individuals were captured using mist-nets placed in two different ringing stations: Scrivia River Valley (N = 147) (province of Alessandria, 44.8087 N, 8.8572 E) and San Genuario Marsh reserve (N = 21) (province of Vercelli, 45.2175 N, 8.1777 E). These locations were selected based upon the high ecological richness and the abundance of mosquitoes (Pollono et al., 1998). Several bird species often breed in both locations, and, remarkably, the Scrivia River Valley is along one of the main migratory paths between Europe and Africa (Silvano and Boano, 2008). Captured birds were identified according to species, sex and age class (Spina and Volponi, 2008a). Birds were then ringed, sampled and released. Other blood samples (N = 136) were made available by the C. A. N. C. (‘‘Centro recupero animali non convenzionali’’), a wildlife rehabilitation center at the Department of Veterinary Sciences, University of Turin, which hospitalizes rescued birds from several areas of NW Italy. Samples origin is shown in Fig. 1B.

Birds were classified in one of the following three groups according to their migratory habits, as indicated in Table 1A and B: residents, short-distance migrants and trans-Saharan migrants (Spina and Volponi, 2008a,b). For those species with a mixed behavior, prevalence data were treated independently and attributed each time to one and to the other behavior group.

2.2. Sampling procedure

Blood samples were collected by syringes or capillary tubes for micro-hematocrit from the brachial or jugular veins according to species. The volume of collected blood never exceeded the 1% of body mass (McGuill and Rowan, 1989). Samples were allowed to clot at room temperature and then centrifuged for 10 min at 5600 g for Eppendorf tubes (Eppendorf Srl Milan, Italy) and 5 min at 3500 g for micro-hematocrit tubes. Sera were stored at -20°C until use.

2.3. Laboratory tests

2.3.1. Virus strains

USUV strain 939/01 isolated from a blackbird in Vienna (Austria) in 2001 and WNV strain Eg-101 were kindly donated by Prof Zdenek Hubalek (Medical Zoology Laboratory, Institute of Vertebrate Biology, Academy of Sciences, Valtice, Czech Republic) and the Unité des Arbovirus et des Fièvres hémorragiques, Institut Pasteur, Paris (France), respectively. The two viruses are routinely used for the diagnostic activities at the Istituto Zooprofilattico Sperimentale of Teramo.

2.3.2. Serological investigation

A total number of 304 serum samples were tested by serum-neutralization (SN) assay according to a recent protocol developed by our group (Di Gennaro et al., 2014). The small volume of some sera also influenced the diagnostic pipeline. In particular, out of 304 samples, 233 and 297 serum samples were tested for the presence of WNV and USUV neutralizing antibodies, respectively. Of these, 226 samples were tested simultaneously for WNV and USUV. In few cases (N = 34) the serological screening for USUV was performed starting at 1:20 dilution of the tested serum.

2.3.3. Molecular detection of WNV and USUV

Two real-time RT-PCR assays were employed for the molecular detection of WNV (Del Amo et al., 2013) and USUV (Cavrini et al., 2007). Nucleic acids were purified from the blood samples of serological positive birds by means of BioSprint 96 One-For-All Vet Kit (QIAGEN, Germany).

3. Results

Results of the serological analyses are shown in Table 2. Out of 297 samples tested for USUV, four had specific neutralizing antibodies (1.34%; 95% IC 0.36–3.4). Antibodies were detected from a blackbird (*T. merula*), a long eared owl (*Asio otus*), a rock pigeon (*Columba livia*) and from a mallard (*Anas platyrhynchos*), sampled at the municipalities of Villarvernia, Scalenghe, Grugliasco and Castagnole Piemonte, respectively (Fig. 1C). Neutralising titers ranged from 1:10 to 1:40. The prevalence of USUV sero-reactors was similar despite the migratory habits of the sampled birds. However, USUV antibodies prevalence may have been underestimated as the serological screening of some sera started at 1:20 dilution.

Out of 233 serum samples tested for WNV, ten had neutralizing antibodies (4.29%; IC 95% 2.07–7.75). The positive samples belonged to a Eurasian scops owl (*Otus scops*), two little bittern (*Ixobrychus minutus*), one nigthingale (*Luscinia megarhynchos*), one Eurasian hobby (*Falco subbuteo*), one European green woodpecker (*Picus viridis*) and one common kestrel (*Falco tinnunculus*), captured in the municipalities of Vercelli, Buriasco, Villarvernia, Trofarello and Villarpellice, respectively (Fig. 1D). The neutralizing titers ranged from 1:10 to 1:80. Neutralizing antibodies for WNV were significantly more prevalent ($p < 0.001$) in trans-Saharan migrants (P = 21%, IC 95% 9.55–37.3, N = 38) than in resident (P = 1.06%, IC 95% 0.02–5.78, N = 94) or short-distance birds (P = 0.99%, IC 95% 0.02–5.39, N = 101). Serological positive samples for WNV (10) and USUV (4) belonged to the 226 serum samples tested by SN for both viruses. Cross-

reactions have not been detected. WNV or USUV serological positive animals turned out to be negative by molecular assays for WNV and USUV RNA.

4. Discussion

In the present manuscript, the exposure to USUV and WNV viruses was investigated in several wild bird species in Piedmont region, northwestern Italy (NW). At the time the present surveillance in wild birds was ongoing (March 2012–June 2013), WNV was never demonstrated to circulate in Piedmont region, and only 3 pools collected during 2009 and 2010 of USUV-positive *Culex pipiens* were reported in a peripheral eastbound area of the region (Cerutti et al., 2012, Fig. 1C). Therefore the aim of the study was primarily to set up a surveillance network based on serological investigation of blood samples collected from live wild birds in order to explore the prevalence of WNV and USUV antibodies in that area.

The scenario slightly changed in the following years. Indeed, WNV was revealed in the region by molecular methods in pools of *C. pipiens* at the end summer of 2014 confirming therefore the circulation of WNV as suggested in the present study (Dr. Cristina Casalone, Istituto Zooprofilattico of Piemonte, Liguria and Val d'Aosta, http://promedmail.chip.org/pipermail/promed/2014-November/005857.html, Fig. 1D). Moreover, a recent paper (Rizzo et al., 2014) describes the evidence of USUV circulation within mosquitoes in Piedmont region (Novara province) during 2011 and 2012 (Fig. 1C). This area is in very close proximity to the Ticino river natural park, a wet area and an important wintering site for wild aterfowl. This recent data and the results produced in this study confirm the establishment of an enzootic cycle of USUV in the Piedmont region.

This survey revealed antibodies against WNV and USUV in resident bird species also. Blood samples from serological positive animals were further screened by molecular assays in order to detect active viraemia. However, none of them showed WNV or USUV RNA in the bloodstream. It was not possible to test the remaining samples as for the limited amount of material to be processed for RNA purification. Reasonably, our study shows some limitations since we were not able to demonstrate active circulation of WNV and USUV in the sampled wild birds. However, the present serological investigation combined with the detection of WNV or USUV RNA from mosquito pools described by other research groups dynamically working on flavivirus circulation in Piedmont region, strongly suggests active circulation of both viruses.

It is assumed that USUV has been introduced in Europe by trans-Saharan migratory species and that the virus adapted over the time to the local environment by establishing a transmission cycle among local birds and mosquitoes (Chvala et al., 2007). On the other hand, the transmission within European countries may have occurred by short-distance migratory birds (Steinmez et al., 2011). Alternatively, trans-Saharan migratory birds may be responsible for the introduction of USUV every year in Europe (Buckley, 2003). The results of this study are in contrast with the second assumption, as no migratory birds tested positive for USUV antibodies and all positive birds were resident or short-distance migrants. Regarding the introduction of WNV, our study supports the scenario of a mixed system in which long-distance migrant birds are primarily involved in spreading WNV from Africa to Europe whereas resident and short distance migratory birds

contribute, afterwards, to the establishment of local enzootic cycles (Savini et al., 2013; Monaco et al., 2011).

Though USUV exposed birds were scattered over a relatively wide area (Fig. 1C), the prevalence of sero-reactors suggests a limited circulation of the virus. Nevertheless, the 1.34% prevalence found is within the range reported from wild birds in Italy (0– 5.2%) (Lelli et al., 2008; Savini et al., 2011). So far, USUV outbreaks with fatal neurological outcome in wild birds have not been reported in NW Italy. In general, the antibody titers against USUV were low, ranging between 1:10 and 1:40, but in naturally and experimentally infected birds, flaviviral antibody titers are generally low and prone to considerable variations within short time (Savini et al., 2011). We may speculate on the putative locations where seropositive birds were exposed to USUV, on the basis of their migratory or sedentary behavior. Two scenarios can be indeed estimated for the juvenile common blackbird sampled in late summer: it may have been exposed while residing in the study area or, alternatively, the presence of specific USUV antibodies derived from passive transfer of maternal immunity, as previously described for WNV (Gibbs et al., 2005). The exposure site of the long eared owl is questionable, since individuals captured in NW Italy are partly breeding or transient (Fasano et al., 2005). The remaining USUV sero-positive birds were one mallard and one rock pigeon. Mallards reside in most of the European countries except in the North and East of Europe, from where they migrate during the winter season (Spina and Volponi, 2008a). Migratory individuals usually arrive in the Piedmont region from northern Europe in November lasting until February-March (Fasano et al., 2005). Possibly, the sero-positive mallard was a resident individual that became infected in NW Italy as the capture occurred in April when wintering mallards are usually gone. However, the presence of USUV antibodies in a rock pigeon, a resident species, suggests a local circulation of the virus.

Antibodies against WNV were detected in two little bitterns sampled few weeks before migration period toward wintering areas. One of them had a high antibody titer (1:80) suggesting a local recent exposure to WNV. High antibody titers (1:80) were also revealed in the nightingale and in one of the Eurasian scops owls. Their arrival in NW Italy from the wintering regions usually occurs in April-May (Fasano et al., 2005). Since both birds were sampled in May, it is reasonable to assume that they were exposed prior to migrate to Europe. The common kestrel was the only short distance migrant among the WNV sero-reactors, but in NW Italy there are also described to be as resident individuals (Fasano et al., 2005). Because of this, the exposure site of the kestrel is difficult to identify. The positive European green woodpecker, a resident species, is a further evidence of a local WNV circulation, as demonstrated elsewhere in other Italian regions (Savini et al., 2013). Significant higher WNV sero-prevalence was detected in trans-Saharan migrants, in accordance with other surveys (López et al., 2008). This difference suggests that long-distance migrants are exposed during the journeys and/or during the winter stay in Africa to a greater magnitude of WNV circulation as compared to the breeding grounds in Europe.

Overall, antibody titers against WNV and USUV detected in wild birds were similar to those regularly observed during experimental infections in birds with WNV (Del Amo et al., 2014; Pérez-Ramírez et al., 2014). It is noteworthy that a slightly lower (1:10–1:40 titer range) neutralizing antibody response was detected for USUV with respect to the neutralizing antibody response observed in those wild birds found serologically positive for WNV showing titers ranging from

1:10 to 1:80. This slight difference in antibody titers could be consistent with a less intense infection by USUV compared with WNV in wild birds.

This study offers a further evidence of the local circulation of WNV and USUV within wild bird species in the NW of Italy. We argue that sampling of live birds allowed a better representation of both viruses distribution for two reasons. First, our survey evaluated a wide range of potential USUV and WNV amplifier hosts mostly dwelling in rural and sylvatic habitats. The chance to detect an emerging infection in these habitats through passive surveillance is low compared with urban areas (Marra et al., 2004), even more so when lethality of circulating viruses is low or when herd immunity is present (Buckley, 2003). Second, surveillance national plans based on both active and passive surveillances are primarily focused on providing direct evidence of early viral circulation (Italian Ministry of Health, 2008) by introducing sentinel fowl in a given area supported by the entomological surveillance and by a very limited number of resident synanthropic wild birds mostly originating from urban areas.

Results of this survey prove that ringing organizations and wildlife rehabilitation centers may be fruitfully involved during the surveillance of bird-related emerging or re-emerging pathogens, as already previously stressed (Nemeth et al., 2007). This – mostly voluntary – network is widespread in Europe (Euring, 2013) and may be potentially very active. In NW Italy, for instance, 44 accredited ringers had worked in 116 stations during 2012. They handled in total 35,660 wild birds belonging to 151 different species (Fasano et al., 2013). In addition, one of the six regional wildlife rehabilitation centers present in the Piedmont region provided samples for the present investigation thus giving evidence of their potential role for a robust surveillance plan assessment. In a public health perspective, the contribution of this network to WNV surveillance would be of particular interest in areas which are not officially infected and/or are close to endemic areas. Data from this network would be of great value to reveal additional information upon WNV and USUV circulation and subsequently address other costly and labor-intensive actions specifically targeted to human health protection. Investigations on the occurrence, ecology and epidemiology of these two important zoonotic viruses are paramount. In this perspective, our work further highlights the need for the establishing of tight connections between veterinarians, physicians, entomologists, public health officers and dynamic voluntary networks.

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Fig. 1. Map of Italy highlighting the geographical position of Piedmont region (A). Map of Piedmont region showing the geographical origin of the samples (B). Geographical origin of USUV positive samples: municipalities of Villarvernia (VV), Scalenghe (S), Grugliasco (G) and Castagnole Piemonte (CP) (C) and positive samples for WNV: municipalities of Vercelli (VE), Buriasco (B), Villarvernia (VV), Trofarello (T) and Villarpellice (VP) (D). : Geographical location of mosquitoes' pools positive for USUV (B) and WNV (C) RNA demonstrated in 2009/2010, 2012 and 2014.

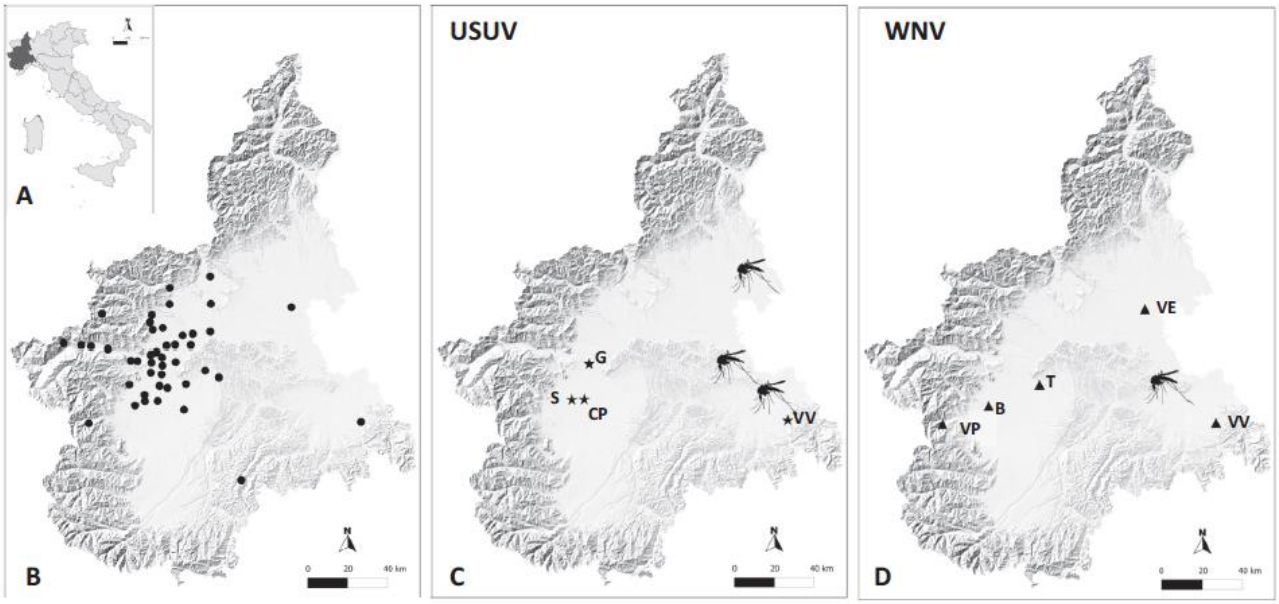

Table 1. (A) and (B) Species, number of sampled individuals tested for USUV and WNV. Each species was classified according to their migratory behavior in resident (R), short-distance migrant (M) or trans-Saharan migrant (T). Birds were divided in two tables, (A) and (B), according to the alphabetical order.

Table 1A

	Migratory behavior	Total samples	Number of samples tested for WNV	Number of samples tested for USUV
Accipiter gentilis	R	2	1	2
Accipiter nisus	M	1	1	1
Alcedo atthis	R - M	1	0	1
Anas platyrhynchos	R - M	6	4	6
Anser anser	M	1	1	1
Apus apus	T	1	1	1
Ardea cinerea	M	3	3	3
Asio otus	M	5	4	5
Athene noctua	R	3	2	3
Botaurus stellaris	M	1	1	1
Bubo bubo	R	1	1	1
Buteo buteo	M	7	7	7
Cairina moschata	R	2	2	2
Caprimulgus europaeus	M	3	2	2
Chroicocephalus ridibundus	M	4	4	4
Columba livia	R	45	41	45
Columba palumbus	M	4	4	4
Corvus cornix	R	23	22	23
Corvus monedula	M	1	1	1
Coturnix coturnix	T	1	1	1
Cygnus olor	M	1	1	1
Egretta garzetta	R - M	2	1	2
Erithacus rubecula	M	1	0	1
Falco subbuteo	T	1	1	1
Falco tinnunculus	R - M	6	3	6

Table 1B

	Migratory behavior	Total samples	Number of samples tested for WNV	Number of samples tested for USUV
Garrulus glandarius	R - M	7	7	7
Ixobrychus minutus	T	6	3	6
Lanius collurio	T	2	2	2
Larus michahellis	R - M	2	1	2
Luscinia megarhynchos	T	3	1	3
Merops apiaster	T	20	10	18
Otus scops	T	18	10	18
Parus major	R - M	6	3	5
Passer italiae	R	1	1	1
Phoenicurus phoenicurus	T	3	1	3
Picoides major	R - M	5	3	5
Pica pica	R	15	14	15
Picus viridis	R	4	3	4
Pyrrhocorax pyrrhocorax	R	1	1	1
Strix aluco	R	3	3	3
Streptopelia decaocto	R	4	3	4
Streptopelia turtur	T	8	7	8
Sturnus vulgaris	M	38	32	38
Sylvia atricapilla	R - T	2	0	2
Tachymarptis melba	T	1	1	1
Turdus merula	M	28	18	25
Turdus philomelos	M	1	0	1
Total number		304	233	297

Table 2

Titers and migratory behavior for individual birds with USUV and WNV neutralizing antibodies sampled between March 2012 and June 2013 in Piedmont, Italy. Each species was classified as resident (R), short-distance migrant (M), or trans-Saharan migrant (T).

	SN USUV POSITIVES								SN WNV POSITIVES								Sample date
		Titres								Titres							
Species	n	10	20	40	80	160	320	640	n	10	20	40	80	160	320	640	
Anas platyrhynchos	6	1							4								April 2012
Asio otus	5		1						4								May 2012
Columba livia	45	1							41								August 2012
Falco subbuteo	1								1			1					June 2012
Falco tinnunculus	6								3		1						April 2013
Ixobrychus minutus	6								3	1			1				August 2012
Luscinia megarhynchos	3								1				1				May 2013
Otus scops	18								10		3		1				May 2013- May 2013, May 2012, May 2013
Picus viridis	4								3				1				May 2013
Turdus merula	25			1					18								August 2012